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ORIGINAL PAPER

The importance of solvent type in estimating antioxidant properties of phenolic compounds by ABTS assay

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Abstract ABTS assay belongs to the most popular methods employed for estimating antioxidant activity. However, researchers seldom pay attention to specific factors influencing the determination of antioxidant activity of the examined compounds and mixtures. The paper shows that the type of alcohol used significantly influences the estimation of antioxidant activity of phenolic compounds in ABTS assay, namely that their antioxidant activity increase with the lengthening of the aliphatic chain in alcohol. It results rather from the changes in $ABTS^{\bullet+}$ solvatation energy by the employed alcohols than from dissociation variations of phenolic compounds. The obtained results point to the difficulties in the correct estimation of the real antioxidant properties of plant and food extracts by ABTS assay. The presented results have also an ecological implication as they refer to the difference in estimation of antioxidant properties of compounds resulting from the replacement of toxic methanol by GRAS (Generally-Recognized-As Safe) solvents, ethanol and propanol.

Keywords $ABTS^{\bullet+}$/phenolic compound reaction kinetics · Antioxidant activity · Alcohol type · Water content

Introduction

Free radicals are responsible for food decay and cause oxidative damages to biological systems [1–5]. For these reasons, oxidation processes and free radicals in living organisms have gained increased attention in the recent decades. Human, animals and plants are continuously exposed to free radicals. They are generated not only in normal physiological processes, for example during mitochondrial respiration, but also are produced by exogenous sources such as radiation and pollutants [6]. In the struggle with free radicals, a living organism is supported by substances called antioxidants which neutralize these reactive species [7–9]. Several methods are used to estimate the antioxidant properties of compounds and extracts [10, 11]. In most of them, the ability of an antioxidant to trap free radicals is measured. The methods employing chromogen compounds are commonly applied due to their ease, speed and sensitivity [12, 13]. ABTS assay, in which the radical cation ($ABTS^{\bullet+}$) derived from 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) is used, seems to be most popular [14, 15]. As $ABTS^{\bullet+}$ reacts rapidly with almost every lipophilic and hydrophilic antioxidant, it is applied to examine both lipophilic and hydrophilic substances, and food products for their antioxidant properties. The idea of the method is to monitor the decrease in $ABTS^{\bullet+}$ absorbance. This chromogen compound exhibits strong absorption in the range of 600–750 nm and can be easily determined spectrophotometrically in any laboratory. The method's additional advantage is its applicability in a wide pH range [16]. Due to the mentioned attributes, ABTS assay is used by many searchers, who believe it allows for an easy, fast and reliable determination of antioxidant properties of the examined compounds.

β-carotene bleaching assay is another method applied for the determination of antioxidant activity of compounds and extracts. It has been demonstrated [17] that the reaction kinetics between an antioxidant and peroxyl radicals in this method depends on the solvent type which account for the estimated antioxidant properties of the examined

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compounds. A question appears whether solvent type also influences the estimation of antioxidant properties of compounds by ABTS assay. The present paper answers this questions by showing and discussing the influence of the type of alcohol on the evaluation of antioxidant activity of butylhydroxytoluene (BHT), used here as standard antioxidant. The alcohols chosen for the experiments are those most frequently used as extracting agents in extraction processes. The present investigations elaborate on the recent results [18] proving that metal ion type and concentration, water content and pH of the measuring system all significantly influence the estimation of antioxidant activity in ABTS assay and thus make the correct estimation of the real antioxidant properties of plant and food extracts difficult. Despite the great popularity of the ABTS method, little is known about the factors influencing the kinetics of the $ABTS^{\bullet+}$/antioxidant reaction, that is, about the factors affecting the estimation of antioxidant activity of the examined compounds by this method.

Experimental section

Reagents

Methanol, ethanol, propanol-1 (all of analytical-reagent grade) and Karl Fischer reagent were purchased from the Polish Chemical Plant—POCh (Gliwice, Poland). 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate (di-potassium peroxydisulfate), 2.2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) and BHT were purchased from Sigma-Aldrich (Poznań, Poland). Water was purified on a Milli-Q system from Millipore (Millipore, Bedford, MA, USA).

Methods

ABTS assay

Generation of ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium] radical cation was performed by Nenandis [16]. The $ABTS^{\bullet+}$ solution was prepared by reaction of 5 ml of a 7 mM aqueous ABTS solution and 88 µl of 140 mM (2.45 mM final concentration) potassium persulfate ($K_2S_2O_8$). The mixture was incubated in the dark for 16 h. The radical cation formed in this way was further diluted in methanol or ethanol or propanol-1 or propanol-2. Two types of alcoholic $ABTS^{\bullet+}$ solutions were applied:

(a) exhibiting the initial absorbance value of 0.71 at 744 nm and
(b) possessing the same amount of $ABTS^{\bullet+}$ (80 or 120 µl of the radical cation solution in 10 ml of the final alcoholic solution).

The $ABTS^{\bullet+}$/BHT reaction kinetics was estimated measuring the decrease in ABTS radical cation concentration at 744 nm (at 757 and 412 nm occasionally) and/or the concentration increase in the reduced ABTS form at 346 nm. To zero the spectrophotometer, pure solvents (free of $ABTS^{\bullet+}$ and BHT) were used. The $ABTS^{\bullet+}$ solutions without antioxidant were applied as controls.

The ABTS assays were performed according to the following procedure: 2000 µl of $ABTS^{\bullet+}$ solution in a given alcohol was mixed in a 4-ml test tube with 20 µl of BHT solution in the same alcohol (0.5 mg/ml). The mixture was stirred vigorously for 30 s and poured into quartz cuvettes (1 cm × 1 cm × 3.5 cm). The changes in absorbance were monitored at a mentioned wavelength for 60 min (180 min occasionally) using a UV Probe-1800 spectrophotometer (Shimadzu, Kyoto, Japan). Subsequent readings were taken at regular intervals (60 s).

The percent of remaining $ABTS^{\bullet+}$ was calculated from the following equation:

$$\% \text{ Remaining} = \left(\frac{A_t}{A_{t_0}}\right) \times 100\ \%$$

where A_{t0} and A_t are the values of absorbance of $ABTS^{\bullet+}$ at 0 min and at time equal to (t) min, respectively.

The $ABTS^{\bullet+}$/BHT reaction kinetics was also estimated observing the concentration increase in the reduced ABTS form at 346 nm. Due to influence of the alcohol type on the intensities of the absorption band at this wavelength, the changes in ABTS concentration were expressed as relative concentration changes. Relative ABTS concentration was calculated from the following equation:

$$\text{Relat.increase} = \frac{A_t - A_{t0}}{A_{t180} - A_{t0}}$$

where A_{t0}, A_t and A_{t180} are the values of absorbance of ABTS at 0 min, at time equal to (t) min and at 180 min, respectively.

Water determination by Karl Fischer

A portion of the examined alcohol (100 µl) was injected into a semi-automatic Karl Fischer-device from Metrohm (Herisau, Switzerland). The results obtained reflected the absolute water content in % of the injected solution.

Statistical analysis

As was mentioned above, the changes in absorbance were monitored for 60 min (180 min. occasionally) at regular

intervals (60 s). The kinetic curves were drown using all experimental data; however, only a few experimental points (mean values ± SD) were put in the figures for their clarity. In order to determine the measurements repeatability, each antioxidant activity assay was done three times. RSD of all measurements were lower than 10%. $P < 0.01$ was assumed as statistical difference between mean experimental points. All statistical analyses were performed using Statistica version 7.0 software package (Statsoft, Tulsa, USA).

Results and discussion

Figure 1 shows the concentration changes of remaining $ABTS^{\bullet+}$ during 60 min of the $ABTS^{\bullet+}$/BHT reaction carried out in systems differing in the type of applied alcohol (methanol or ethanol or propanol-1). The measuring systems contained the same amounts of reagents and solvents, and the measurements were performed according to the method described elsewhere [16]. As results from the established relationships, the concentration of unreacted $ABTS^{\bullet+}$ after a 60 min reaction is different and depends on the alcohol type. The fastest kinetic of $ABTS^{\bullet+}$/BHT reaction is observed for propanol-1 and the lowest for methanol. It should be noticed that the applied alcohols contained different amounts of residual water (see legend in Fig. 1). According to Dawidowicz and Olszowy [18], the kinetics of the $ABTS^{\bullet+}$/BHT reaction strongly depends on water content in the measuring systems as the increase in water concentration in the methanolic system causes the increase in the $ABTS^{\bullet+}$/BHT reaction rate. Hence, the presented differences in $ABTS^{\bullet+}$/BHT reaction kinetics (Fig. 1) can be assigned to different water concentrations in the used alcohols. The comparison of the relation between kinetic curves in Fig. 1 with the water concentration in the applied alcohols confirms the influence of water, but only in the case of ethanol solutions. Figure 2 illustrates the difference (Δ) in the amount of unreacted $ABTS^{\bullet+}$ in the systems containing initial methanol or ethanol or propanol-1 and in the same solvents at different amounts of water. In other words, it presents the influence of water concentration in a given alcohol on the acceleration of the $ABTS^{\bullet+}$/BHT reaction kinetics. As water concentration in initial ethanol in relation to methanol and propanol is significantly smaller, for better illustration of water influence on the difference in reaction kinetics (Δ), Fig. 2 additionally shows the dependence obtained when ethanol containing about 1.5 % of water was used as relative solvent (99.8 % ethanol was enriched in water). The results corresponding to methanol and ethanol agree with previous data [18]. In the case of propanol, the initial increase and than the decrease in $ABTS^{\bullet+}$/BHT reaction rate with water addition

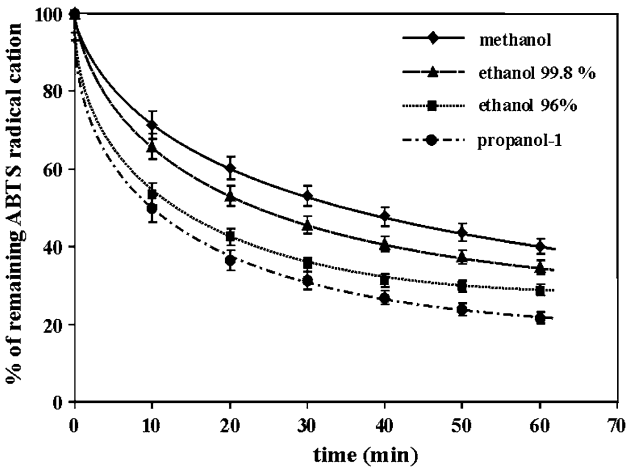

Fig. 1 The influence of alcohol type (methanol, ethanol and propanol-1) on the kinetics of $ABTS^{\bullet+}$/BHT reaction curried out during 60 min

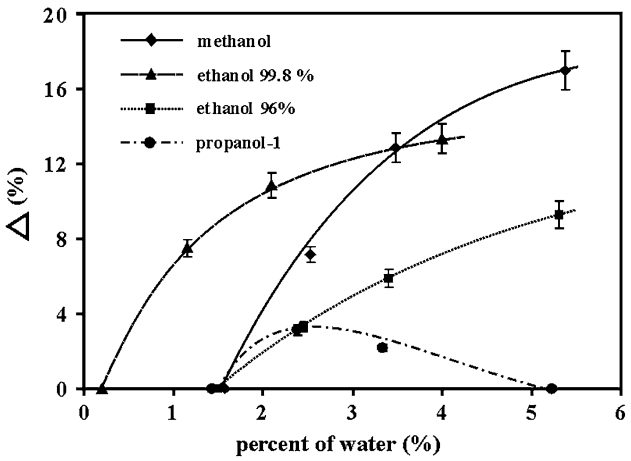

Fig. 2 The influence of water concentration in methanol, ethanol and propanol-1 on the difference (Δ) in the amount of unreacted $ABTS^{\bullet+}$ in the systems containing the initial alcohol and in the same solvents at different amounts of water

is observed in the examined concentration range. Moreover, the influence of water content on the kinetics of $ABTS^{\bullet+}$/BHT reaction is stronger for methanolic systems and weaker for propanolic systems. Hence, the differences in the concentration of water in the used alcohols do not explain the influence of alcohol type on the $ABTS^{\bullet+}$/BHT reaction velocity.

The results presented in Fig. 1 were obtained following the general assumption for ABTS assay that the absorbance of the initial ABTS radical cation solution should equal 0.71±0.05 at 744 nm. It needs mentioning at this point that different volumes of $ABTS^{\bullet+}$ stock solution have to be applied to reach the required initial absorbance of individual alcoholic solutions. For instance, in the presented experiments, about 150 or 120 µl volumes of

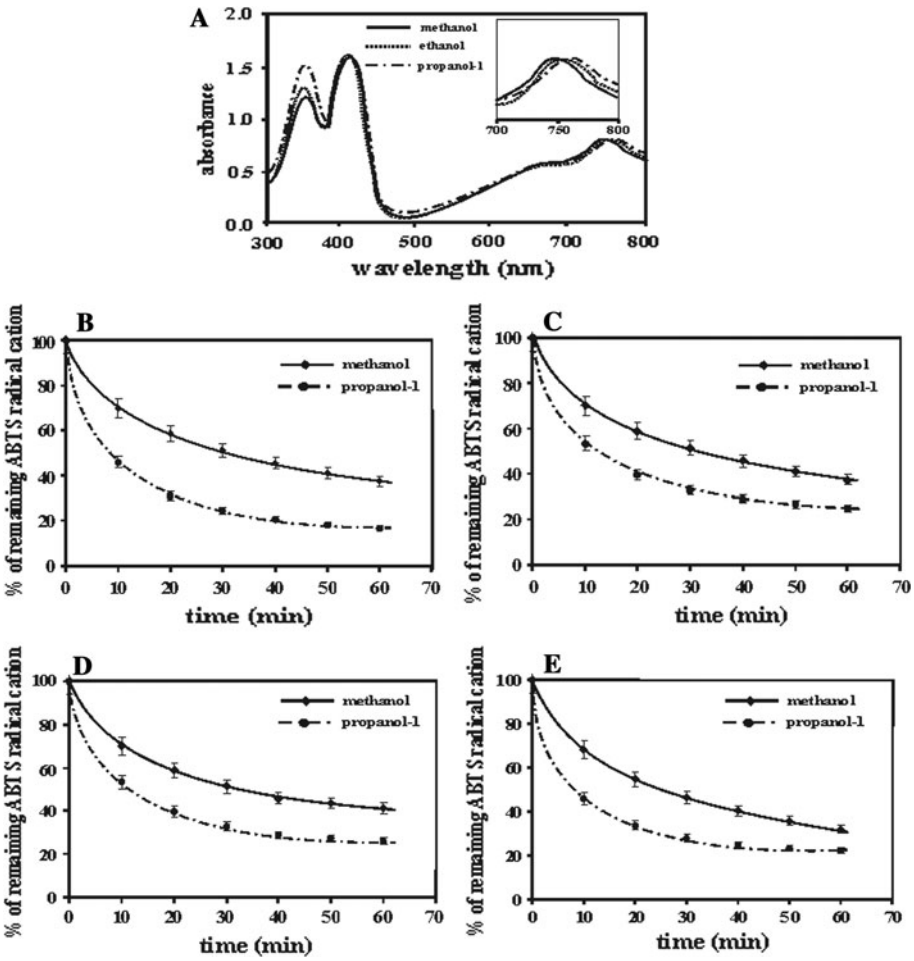

Fig. 3 Absorption spectra of ABTS and its oxidation product, $ABTS^{\bullet+}$, in methanol, ethanol and propanol-1 (**a**), and the influence of alcohol type (methanol and propanol-1) on the concentration changes of remaining $ABTS^{\bullet+}$ during 60 min of $ABTS^{\bullet+}$/BHT reaction measured: at 744 nm for methanol and at 757 nm for propanol-1 in systems with the initial absorbance of ABTS radical cation solution equal to 0.71 (**b**); at 744 nm for methanol and at 757 nm for propanol-1 in systems containing the same volumes of $ABTS^{\bullet+}$ stock solution (80 µl) (**c**); at 744 nm for methanol and at 757 nm for propanol-1 in systems containing the same volumes of $ABTS^{\bullet+}$ stock solution (120 µl) (**d**); at 412 nm (**e**)

stock solution were used to prepare 10 ml volumes of propanolic or methanolic solution, respectively. All this suggests the solvatochromic effect in the $ABTS^{\bullet+}$/ABTS spectrum. The spectra of $ABTS^{\bullet+}$/ABTS in methanol or ethanol or propanol are presented in Fig. 3a. The absorption maxima at about 412, 658 and 744 nm correspond to ABTS radical cation, and the maximum at about 346 nm corresponds to the reduced ABTS form [19, 20]. As results from Fig. 3a, the replacement of methanol with propanol-1 shifts the maximum of the recommended absorption band from 744 to 757 nm. Figure 3b–e shows the influence of alcohol type on the concentration changes of remaining $ABTS^{\bullet+}$ during 60 min of $ABTS^{\bullet+}$/BHT reaction measured:

- at 744 nm in the case of methanol and at 757 nm in the case of propanol-1 for systems with the initial absorbance of ABTS radical cation solution equal to 0.7;
- at 744 nm in the case of methanol and at 757 nm in the case of propanol-1 for systems containing the same volumes of $ABTS^{\bullet+}$ stock solution (80 and 120 µl);
- at 412 nm.

These experiments were limited to the comparison of the systems with methanol and propanol-1 due to the observed distinct differences in kinetics of $ABTS^{\bullet+}$/BHT reaction carried out in these alcohols. The dependences presented in Fig. 3B-3E confirm the finding from Fig. 1—the $ABTS^{\bullet+}$/BHT reaction rate is faster in propanol-1 than in methanol. Hence, it can be concluded that the observed difference in reaction rate does not result from solvatochromic effect of $ABTS^{\bullet+}$/ABTS system.

Many authors [21, 22] classify ABTS as an electron-transfer method (ET). The ET-based method detects the ability of a potential antioxidant to transfer one electron to reduce a radical (e.g., $ABTS^{\bullet+}$). According to Musialik & Litwinienko [23], this process is accelerated in a medium supporting the ionization of the antioxidant. Hence, the obtained results may suggest better ionization of BHT in propanol-1 than in methanol. Yet this supposition may raise some doubts. Many pKa values for phenolic compounds in alcoholic solutions are found in literature [24]. While some of them indicate increasing ionization of phenols when their solvent is changed from methanol to

propanol, most of them show the opposite dependence. Hence, the observed increase in the $ABTS^{\bullet+}$/BHT reaction velocity resulting from replacing methanol with propanol is difficult to explain by the increase in BHT ionization.

The influence of the alcohol type on the $ABTS^{\bullet+}$/BHT reaction kinetics may be connected with the structural differences of bulk alcohols. Bulk alcohols are composed of hydrogen-bonded clusters [25, 26] that remain in thermodynamic equilibrium. According to Borowski et al. [25], bulk methanol is mostly composed of hepta-, hexa-, penta-, tetra- and trimeric cluster structures. Monomeric methanol molecules constitute only a minor fraction of bulk alcohol. The amount of the isolated alcohol molecules is also low in bulk ethanol, but the rest of alcohol is composed mainly of pentameric clusters at room temperature. It cannot be excluded that various cluster structures of the used alcohols exhibit a different ability of proton transmission and, consequently, are responsible for the observed differences in the reaction kinetics.

These differences can also result from the solvatation energy of the used radicals ($ABTS^{\bullet+}$) in the employed alcohols. It is probable that more polar and more acidic methanol molecules strongly interact with $ABTS^{\bullet+}$(especially with the electron pairs existing at the =N–N= bridges of these radicals), inhibiting both the electron and/or hydrogen atom transfer between the antioxidant and these radicals.

As results from the literature [27], $ABTS^{\bullet+}$ reacts with phenolic compounds in two steps. First, one molecule of ABTS radical cation abstracts an electron (or hydrogen atom) from the phenolic compound and forms a semiquinone radical and regenerates the parent substrate, ABTS (reduced form of ABTS). Second, semiquinone radical reacts with another $ABTS^{\bullet+}$ molecule and forms the $ABTS^{\bullet+}$/phenolic antioxidant adduct, which is unstable and degrades to other products. The results presented so far are based on the concentration changes of ABTS radical cation which disappears in the measuring system due to its reduction (step one) and degradation (step two). Thus, the influence of alcohol type on $ABTS^{\bullet+}$-BHT reaction kinetics is observed both in the first and the second step of the reaction. The influence of the solvent type on the kinetics of the first step of the BHT reaction can be examined investigating the concentration changes of the reduced ABTS form, which increases in the measuring system. The absorption band at 346 nm corresponds to the reduced form of ABTS (see Fig. 3a). As results from Fig. 3a, the intensity of this band strongly depends on the alcohol type. The highest intensity is observed for the propanolic solution, and the weakest for the methanolic $ABTS^{\bullet+}$/ABTS solution. The measurement of the reaction kinetics between ABTS radical cation and antioxidant in terms of the concentration increase in reduced ABTS form is not recommended due to strong spectrum fluctuations at 346 nm. Despite this

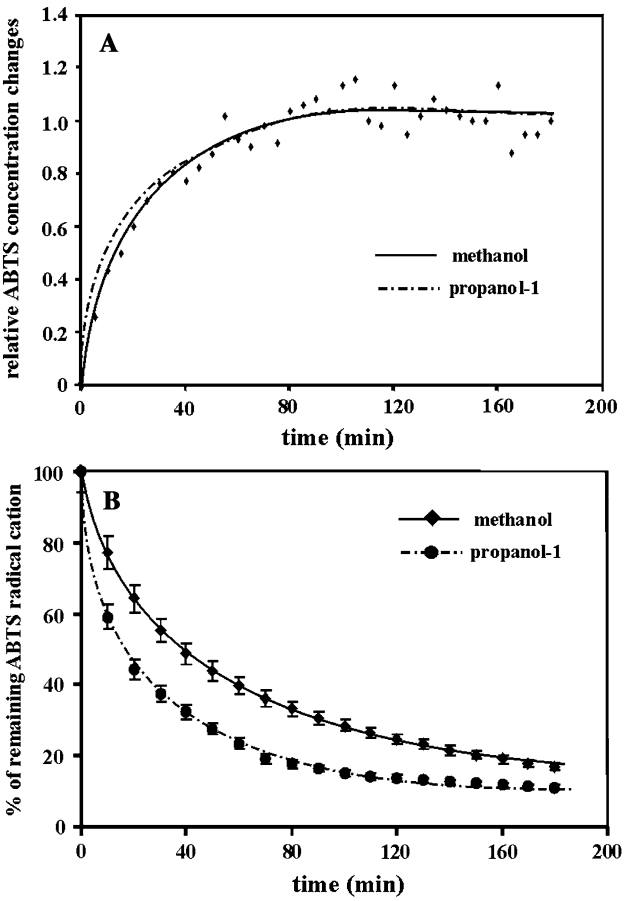

Fig. 4 The influence of alcohol type (methanol and propanol-1) on **a** the relative ABTS concentration changes (measurements at 346 nm), and **b** $ABTS^{\bullet+}$concentration changes (measurements at 744 and 757 nm for methanolic and propanolic solution, respectively) during 180 min of $ABTS^{\bullet+}$/BHT reaction

inconvenience, it was decided to check the influence of the alcohol type on the velocity of the first step of the $ABTS^{\bullet+}$/BHT reaction. Due to the difference in the intensities of the absorption band of the reduced ABTS form, its relative increase in the measuring system is a more convenient illustration of the influence of the alcohol type on the velocity of parent ABTS formation. Figure 4a presents the relative increase in the reduced ABTS form measured during 180 min for the first step of $ABTS^{\bullet+}$ /BHT reaction carried out in systems differing in alcohol type, methanol and propanol-1. The presented dependences (bold lines) were established by averaging the experimental data (sample experimental data are shown only for the system with methanol—thin dotted line), which in crude form was difficult for direct interpretation due to strong fluctuation of 346 nm band intensity. Figure 4b shows the influence of the alcohol type on the reduction in $ABTS^{\bullet+}$ in the same cuvette, that is, it presents the influence of the alcohol type on both $ABTS^{\bullet+}$/BHT reaction steps. The clearly visible influence of the alcohol type on the entire $ABTS^{\bullet+}$/BHT

reaction rate (Fig. 4b) when compared to its influence on the rate of the first reaction step (Fig. 4a) suggests that the second step of the $ABTS^{\bullet+}$/BHT reaction depends mainly on the alcohol type. Hence, out of the two proposed models explaining the influence of alcohol type on $ABTS^{\bullet+}$/BHT reaction rate—cluster structure of alcohols and $ABTS^{\bullet+}$ solvatation process—the second seems to be more reliable because more polar and more acidic methanol molecules strongly interact with $ABTS^{\bullet+}$ (especially with electron pairs existing in the =N–N= bridges of these radicals), inhibiting the formation of covalent adduct ($ABTS^{\bullet+}$/BHT) during the second step of the reaction. According to Valgimigli et al. [28, 29] alcohol cause the change of free spin distribution in DPPH radical. It cannot be excluded that the similar effect occurs in ABTS cation radical.

Conclusions

ABTS assay belongs to the most popular methods employed for estimating antioxidant activity. However, researchers seldom pay attention to specific factors influencing the determination of antioxidant activity of the examined compounds and mixtures. The results discussed in the present paper concern one of the involved factors and show that the solvent change in the measuring system, even not significantly different in physicochemical properties, cause considerable differences in the amount of unreacted $ABTS^{\bullet+}$. The performed experiments indicate that the differences in $ABTS^{\bullet+}$/BHT reaction kinetics result from the changes in $ABTS^{\bullet+}$ solvatation energy connected with the employed alcohols rather than from BHT dissociation variations. However, the impact of bulk alcohol structure on the observed differences cannot be excluded. The presented results have also ecological implication as they refer to the difference in estimation of antioxidant properties of compounds resulting from the replacement of toxic methanol by GRAS (Generally-Recognized-As Safe) solvents, ethanol and propanol.

Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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